THE FORMATION AND STRUCTURE OF THE GLOCHIDIAL CYST[1]

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Introduction

An important phase in the development of fresh-water mussels is the obligatory period of parasitism spent upon appropriate fish hosts. While superficially encysted on such hosts the tiny larval 'glochidium' transforms into a free-living juvenile mussel, more complex in internal structure but without any corresponding increase in external size. The purpose of the present communication is to record the events incident to the formation of the glochidial cyst, to describe the structure of the cyst throughout parasitism, and to examine the morphological relations existing between parasite and host to subserve metabolic functions. A preliminary report was published some years ago (Arey, 1923). Data for the hookless group of glochidia have been drawn chiefly from an intensive study of infections of *Lampsilis luteola* on the gills of the large-mouth black bass (*Micropterus salmoides*), and of *Lampsilis anodontoides* on the long-nosed gar (*Lepisosteus osseus*). Similarly, the hooked series comprised stages of *Hemilastena ambigua* on the gills of the urodele *Necturus*, and stages of *Anodonta corpulenta* on the fins of the orange-spotted sunfish (*Lepomis humilis*).

Closely graded stages of encystment are easily procured by intro-

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ducing the host into a small aquarium into which ripe glochidia have been placed. Attachment follows quickly, and samples of the gills or fins bearing glochidia can then be removed at intervals as desired. Such samples of *L. luteola*, *A. corpulenta* and *H. ambigua* were fixed promptly in Zenker's fluid. The *L. anodontoides* stages were preserved in Bouin. All the material was sectioned serially in paraffin at 6 μ and stained with hematoxylin and eosin.

Attachment of Glochidia

The tiny bivalved glochidium (0.3 mm. or less in size) is incapable of locomotion when liberated from the maternal gill. Chance alone brings it in contact with suitable hosts. If a fin or gill filament becomes inserted momentarily between the valves so that the chimney-like hair cells are touched, the glochidium snaps shut vigorously, pinching the intercepted tissue.[2] The more delicate, hookless group of glochidia attaches to the soft gill filaments, but the sturdy, hooked glochidia can pierce the fins as well. The process of attachment may be observed on excised gill filaments or fins placed with glochidia in a watch glass under the microscope.

Hookless Glochidia.—The sharp edges of the valves cut cleanly through the gill epithelium, affording surprisingly little evidence of hemorrhage or seeping from the incision. The location of the parasite on the gill filament governs the character of the bite. Those that attach to the blade-like edge of the filament cut through the epithelium and, usually, considerable connective-tissue stroma as well.[3] This is characteristic of most well-attached larvae, for these enclose a liberal amount of the deeper tissues. After the epithelium is passed the valves continue to close, cleaving and compressing the underlying stroma. The softer tissues are cut; the tougher gill substance, especially that containing blood vessels, is merely pinched. The walls of the blood vessels and other resistant constituents are constricted at the level of the compression, and expand like an hourglass on either side (Figs. 1 and 2).

It appears that the valve rim cuts both epithelium and soft stroma with ease, but when it encounters the tougher elements, the rim buckles inward until it lies flat against the interior of the valve proper.[4]

[2] Arey (1921). This publication contains a full discussion of the factors involved in closure.

[3] Attachment along the edges of the filament is most favorable for easy and successful encystment. Many glochidia embed deeply in the firm gill substance, sometimes even half below the surface.

[4] The mechanisms involved in the operation of both flange and hook are described in full in a separate contribution (Arey, 1924).

This serves the very practical purpose of furnishing a broad zone of contact, while at the same time the glochidium is prevented from cutting itself entirely free (Figs. 2 to 5).

Attachment to the gill lamellae is essentially similar, but as thin-walled, vascular laminae are encountered in this instance, the chief factor is compression rather than incision. In a typical case the pinched lamellae converge inward toward the approximated valvular rims.

Hooked Glochidia.—The events during the attachment of hooked glochidia are comparable to those already described for the hookless type. Gill infections are practically identical, but fin parasites may lie wholly within the epithelium. The hooks flex much as do the flanges in the other group, but their effect is more local.[4] They pierce the host tissue like tongs, and then are inturned; the tissue is thereby held firmly, while the spines which beset the outside of the hook lock it still further.

Encystment

Generalities.—The process of attachment is completed almost instantaneously. Both incision and compression are accomplished in less than a second. As a result, the ventral edges of the valves sink somewhat below the surface level of the host tissue (Fig. 1). There next ensues a period during which the glochidium is overgrown by the contiguous cellular tissue of the host. Successive stages of this are shown in Figs. 2 to 5. The covering-in process is rapid. In summer the black bass completes its gill cysts in about $3\frac{1}{2}$ hours; yet I have observed fully formed cysts as soon as $2\frac{1}{2}$ hours after attachment, and well advanced stages at one hour. Excised filaments in watch glasses may encyst glochidia even quicker than under normal conditions; two hours has been found sufficient to complete the process. The response in the gar-pike is slower than in the black bass, but encystment has been observed in three hours. Lower temperatures retard the reaction proportionately.

Glochidia which attach to gill lamellae do not form cysts as readily as those on the thicker edges of the filament. This is doubtless due to the amount of material available, as will be explained presently. On the same gill the lamellar cysts may demand twice the time taken by those along the filament's edge. Lamellae adjacent to the glochidium may unite by fusion to form the basal part of the cyst, which is then roofed over in the usual way.

At first, cysts tend to be somewhat thick, irregular and unsymmetrical (Fig. 5). Within two or three days they usually become thinner, smooth contoured and even (Fig. 6). My observations are in

complete agreement with Young (1911) on this point; it is strange that Schierholz (1889), Faussek (1901) and Harms (1907) have all described the cyst as originally thin and only gradually gaining thickness. When glochidia acquire a weak attachment and clasp but a small shred of host tissue, encystment is commonly unsuccessful and the glochidium is lost.

The Method of Cyst Formation.—It is natural to assume that direct proliferation of the cells of the host tissue provides the material for the cyst that encloses the glochidium. Indeed, this assertion is presented as the correct explanation of encystment by several observers (Young, 1911; Lefevre and Curtis, 1912; and still earlier workers). That such an explanation is both inadequate and contrary to fact has been the topic of another publication by the present writer (Arey, 1932*a*). The *a priori* argument against encystment through cell multiplication rests on several facts: (1) The cyst may be composed of several thousand cells; (2) the time required for the formation of a cyst under favorable conditions is only three to four hours; (3) the mitotic cycle is relatively slow and consumes several hours in cold-blooded vertebrates.

Actual observation of encystment stages does not show the presence of more than the ordinary number of random mitoses seen in control, uninfected tissue. For example, in 78 cysts of *Lampsilis luteola* on the black bass, representing stages between 30 minutes and 9 hours after attachment, a total of only 20 positive mitotic figures and 14 doubtful ones were found as the result of a thorough census under the highest magnification. Again, in 17 cysts of *Hemilastena ambigua* on the gills of *Necturus* only one mitotic figure occurred during the period of encystment.[5] These results definitely disprove the theory of encystment through the proliferation of new cells.

Turning now to the real factor underlying encystment, the natural alternative method is actually encountered. This process is one of cell migration, whereby neighboring host cells assemble and actively push forward over the invader until the wound is closed and the glochidium is covered in.[6] After encystment is complete there may be a compensatory period of cell division in the vicinity of the cyst to replace the cells lost during the cellular emigration leading to cyst formation. For the details of this process, and its relation to wound healing in general, the reader is referred to the complete publication already mentioned (Arey, 1932*a*).

[5] My material did not include stages beyond cysts three-fourths completed.

[6] In gill infections on fishes the cyst wall is composed both of epithelium and connective tissue (Figs. 5 to 8). Goblet cells or pigment cells are frequently carried along into the cyst. (Figs. 5 to 8).

The Structure of Cysts

The cyst is repeatedly designated as 'epithelial' by authors who have written on these matters. This, however, expresses only a half truth. Fin parasites, to be sure, may lie entirely within the stratified epithelium, and the same is true for some of the *Hemilastena* cysts on *Necturus* gills. But even in these cases there is commonly attachment to fin rays or connective-tissue stroma which necessitates a more or less extensive defect in the epithelial covering at its base.

Parasites on the gills of fishes usually bite deep into the stroma. Not only does connective tissue adjoin the glochidium here but it is carried up into the roof of the cyst as well, so that the larva in reality lies embedded in stroma (Figs. 2 to 4).[7] Often the epithelium forms a mere external arching canopy. The demarcation between epithelium and cellular connective tissue is commonly very indistinct, and the latter is easily mistaken for the former.[8] Doubtless this circumstance accounts for the existing confusion and erroneous statements concerning the composition of the cyst wall, for in some locations the interpretation is indeed puzzling and the two do appear to blend. Yet

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EXPLANATION OF PLATE

Abbreviations

a.m., adductor muscle of glochidium
c.t., connective tissue of cyst
c.w., cyst wall
ep., epithelium of cyst
f., flange of valve
g.c., goblet cell
g.f., gill filament
gl., glochidium
h.t., host tissue
l.m., larval mantle

Fig. 1. Glochidium of *Lampsilis luteola* just attached to a gill filament of the black bass. Photo. × 150.

Fig. 2. An early stage in the encystment of *L. luteola* (30 minutes after attachment). Photo. × 300.

Fig. 3. A half-formed cyst enclosing *L. luteola* (1¼ hours after attachment). Photo. × 300.

Fig. 4. A cyst nearly completed about *L. luteola* (2¼ hours after attachment). Photo. × 300.

Fig. 5. The complete encystment of *L. luteola* (3½ hours after attachment). Photo. × 300.

Fig. 6. The wall of a *L. luteola* cyst at five days. Photo. × 355.

Fig. 7. The wall of a *L. luteola* cyst at four days. Photo. × 700.

Fig. 8. Tangential section of the wall of a large *L. luteola* cyst on a black bass with acquired immunity (21 hours after attachment). Photo. × 370.

Fig. 9. Gill filament of the black bass. Notches indicate the former location of sloughed *L. luteola* glochidia. Photo. × 19.

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[7] Deep-lying melanophores have been found in the cyst wall. In some cysts, especially those associated with immunity, eosinophils also invade the stroma (Arey, 1932*b*).

[8] Mallory's connective tissue stain does not differentiate these tissues in fishes.


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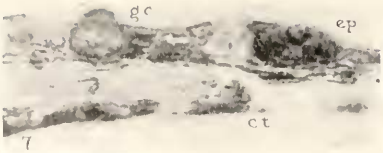
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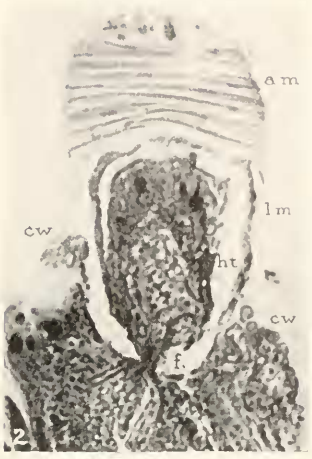
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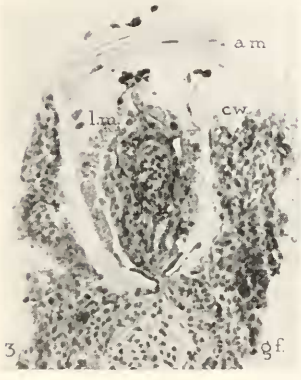
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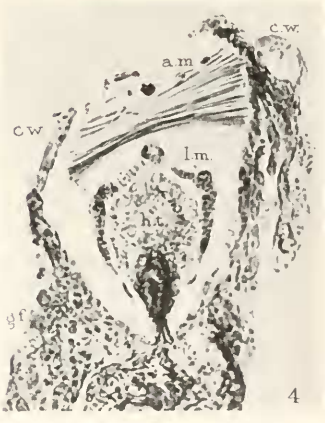
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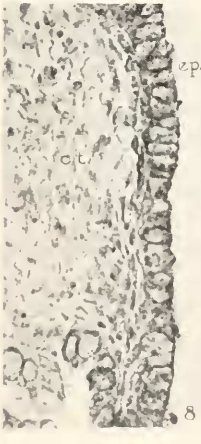
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in favorable preparations the demarcation is clear (Figs. 6 to 8); in the *Lampsilis anodontoides* series on the gar this differentiation was especially evident. Corroborative proof lies in the fact that delicate blood vessels may course through the cellular stroma, and in immune animals eosinophils wander freely through it (Arey, 1932*b*).

Correlative to these findings, the propriety of the term 'cyst' may be questioned as an exact designation for all glochidial investments. If by 'cyst' is meant a distinct envelope which demarks the larva from the adjacent tissues, then such does not exist. In the fin parasites the glochidium simply lies buried in the epithelium, or partly in the connective-tissue stroma. In the gill parasites the position is primarily in the stroma, with a roof-like canopy of epithelium outside. Yet the term is so thoroughly established and convenient as to make its replacement unwise.

The original irregularities of the cyst (Fig. 5) smooth over, and after a few days the roof tends to appear stretched and compact (Figs. 6, 7 and 9). Usually the distinction between epithelium and connective tissue then becomes plainer (Figs. 6 to 8). The goblet and pigment cells carried up in gill infections persist there (Figs. 6 and 7).

Relation of the Glochidium to its Host

Since the glochidium cannot metamorphose except on appropriate hosts it might be thought that special nutritive relations are established between parasite and host, and that this results in recognizable morphological changes or adaptations in the enveloping tissue. As a matter of fact, this possibility is not realized (Figs. 6 to 8). The soft, and for the most part highly vascular, tissues in which the parasite is embedded are apparently adequate for handling whatever interchanges are necessary without any special elaborations. The host tissue ingested at the time of attachment, together with the degenerating larval adductor muscle, are important sources of nutriment during transformation (Arey, 1932*c*), so that there is no metabolic 'strain.' The adjoining host tissues do not become unusually vascularized (Figs. 7 and 8) except in the *Proptera* glochidial type which is peculiar in that it undergoes marked increase in size during a postmetamorphic period of retention. In some specimens of *Proptera laevissima* which had increased in bulk some 40 times, the cysts were found to be very large and thick, and capillaries were present that presumably represented secondary invasive growths.

The Rupture and Repair of Cysts

When the cyst first forms, its wall is regionally variable in thickness and usually bears irregular outgrowths (Fig. 5). After a day or two it

becomes smooth and quite symmetrical. The tissue over the top gradually assumes a compact and stretched appearance, and the wall as a whole is thinner (Fig. 6). This reduction and thinning is much more spectacular in the bulky cysts which characterize the brief attachment of glochidia on immune hosts or non-hosts. In a contribution (Arey, 1932*b*) specifically describing these conditions it will be shown that the thinning is apparently due to the removal of cells back into the filament, rather than to a loss by desquamation or otherwise.

After the first days of encystment there are no especially significant changes in the cyst until the time when the glochidia are shed. Young (1911) has described a characteristic loosening of the cyst tissue and a concomitant infiltration of lymph after about one week of parasitism. A mild degree of cellular separation, in which intercellular bridges become prominent, occurs also in some of my series. Nevertheless, this is by no means a regular phenomenon, while sometimes it is observed relatively distant from an encysted glochidium as well. To what extent such alterations are artefacts and what their proper interpretation may be must remain unanswered at present. Miss Young suggested a causal relation to the premature sloughing of partly transformed glochidia. This may be true, but if so it is not a characteristic method by which these parasites terminate a normal period of encystment.

At the end of the parasitic period the glochidium becomes free of the host.[9] It has not increased in external size, but internally the metamorphosis is marked. Liberation is partly the result of the young mussel's own activity, for at intervals prior to detachment the valves may be observed to move and the foot to be pushed about, pressing the cyst wall. This is demonstrable when at this time filaments are removed and kept in watch glasses under a microscope; incidentally, there is reason to suspect that emergence is accelerated by such *in vitro* procedure. The cyst is eventually ruptured, but sections do not show that this is made perceptibly easier by any sudden terminal thinning or weakening of the wall. Portions of the old cyst-covering may be carried away and adhere for a time to the freed glochidium. Apparently a certain amount of gross sloughing aids the shedding process, for when infections are made on immune fish, transformation of the glochidium fails and the passive glochidium is liberated while still encysted (Arey, 1932*b*).

The freeing of the transformed glochidium leaves a defect in the filament which is rapidly filled in (Fig. 9), probably by the same sort of

[9] Those at the tips of gill filaments are often retained longest (Fig. 9). This is conceivably due to their less favorable position for receiving nutrition or oxygen.

cell mobilization that characterized encystment. Sections are not particularly informative on this point, and even the sites of the cysts are not easily detectable in microscopic preparations. In some instances the cavity of the remnant of the former cyst is temporarily filled with a coagulable exudate.

Summary

Mechanical stimulation of the larval glochidium induces non-selective, automatic closure upon the impinging gill or fin. The valves largely cleave the soft tissues encountered, but merely clasp such tough elements as blood vessels and fin rays which lie deeper. As a result, part of the glochidium is buried in host substance.

The glochidium is then covered by host tissue which advances from all sides, primarily for the purpose of closing the wound. Encystment is not the result of cell proliferation. On the contrary, it is accomplished by a mass movement of cells from the adjoining regions, advancing by their own activities and directed over the exposed valves by thigmotaxis. A compensatory period of mitosis may appear subsequent to encystment, apparently to replace cells lost to the cyst by emigration.

Fin cysts are largely epithelial in structure. Glochidia which attach to gill filaments lie embedded in cellular connective tissue, roofed over with an epithelial canopy.

Shortly after encystment is completed the cyst becomes thinner, smoother and more symmetrical. Thereafter, and even until the time of rupture, there are no further significant morphological changes in the cyst. Special adaptations of the host tissues to care for the wants of the metamorphosing parasite are not developed.

The glochidium is liberated partly through its own efforts, apparently aided somewhat by sloughing. Repair of the resulting defect in the host tissue is rapid and probably follows the general method utilized at encystment. This would involve an early redistribution of existing cellular elements, followed later by the formation of new cells to restore the tissue balance.

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